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13. ABSTRACT ***(Maximum 200 Words)***

Estrogen receptor (ER) is a ligand-activated transcription activator. To elucidate the mechanism of ER-mediated transcription in detail, we studied transcriptional activity of the ER *in vitro*. We demonstrated ER-mediated transcription in a cell-free transcription system is ligand-dependent. Antiestrogens ICI164,384, ICI182,780 and 4-hydroxytamoxifen significantly inhibited transcriptional activity of the ER. Estradiol overcame the inhibitory effect of the antiestrogens and induced ER-mediated transcription. Under the condition used for transcription assays, ICI164,384 and ICI182,780 inhibited ER-ERE complex formation which might contribute to the inhibitory effect of these antiestrogens on ER-mediated transcription. 4-hydroxytamoxifen changed the mobility of ER-ERE complex in the gel mobility shift assay, suggesting a conformational change of the complex. Steroid receptor coactivator 1 (SRC-1) significantly augmented ER-mediated-transcription *in vitro*. The hormone binding domain (HBD) of the ER that binds to estrogen receptor associated proteins (ERAPs) in a ligand-dependent manner inhibited ER-mediated transcription *in vitro*. In contrast, the truncated ER HBD (ERΔ534) lacking of 535 to 595 amino acids of the ER which binds to estradiol but does not associate with ERAPs did not affect ER-mediated effect in this study, suggesting either that ERAPs are required for the full transactivation activity of the ER, or that the amino acid sequence between 535 to 595 is structurally necessary for the ER-mediated function. However, depletion of ERAPs did not significantly affect ER-mediated transcription *in vitro*, indicating that ERAPs might not be essential for ER-mediated transcription *in vitro*.

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Hong Liu 12/29/99

PI - Signature Date

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INTRODUCTION

Estrogen receptor alpha (ER) is a member of nuclear hormone receptor superfamily which includes steroid hormone receptors, thyroid and retinoid hormone receptors, vitamin D receptor, and a large number of so-called orphan receptors for which no ligands have been identified. These receptors function as ligand-activated transcription factors. The ER was identified in 1960s and the function of the ER as a transcription regulator was proposed. Since then, extensive studies have been conducted to probe the detail mechanism of ER-mediated effects at molecular levels. With the cloning of the ER gene, significant progresses have been made in the elucidation of the structure of the ER and the dissection of the mechanism of ER-mediated signal transduction. Human ER is a protein of 595 amino acids and has a molecular weight of approximately 67 kDa. Although different mechanisms have been proposed, most studies on ER-mediated signal transduction have been carried out on the classic ER-ERE pathway. ER binds to estrogen, forms a homodimer, recognizes and binds to a palindromic cognate estrogen receptor responsive elements (EREs) with a consensus sequence of GGTCANNNTGACC which locates in the regulatory regions of ER targeted genes and then regulates gene transcription. However, the detailed mechanism of ER-mediated transcription is still unknown. There is evidence that ER facilitates the formation of the initiation complex. The extensive studies on ER-mediated signal transduction led to the discovery of a set of ER associated proteins (ERAPs) which gives a much more sophisticate view of the mechanism of nuclear receptor action. Up to now, several nuclear receptor associated proteins have been identified or cloned in different laboratories, including ERAP140, RIP140, SRC-1 and related proteins, p300/CBP, SWI2/SNF2, TIF1, and TRIP1. These nuclear receptor associated proteins bound to nuclear receptors in ligand-dependent manners correlates to the ligand-dependent transcription of the receptors in cells, suggesting a putative role of the nuclear receptor associated proteins in ligand-activated receptor-mediated transcription. In this study, a cell-free transcription system was used to study the effects of ER-associated proteins on ER-mediated transcription. We showed that ER-mediated transcription *in vitro* was ligand-dependent under the condition used in this study. ER hormone binding domain associated proteins were required for the full transcription activity of ER. GST-SRC-1 enhanced ER-mediated transcription *in vitro*.

Body

To study the function of the ER *in vitro*, a cell free transcription system was set up as described previously (32, 35).

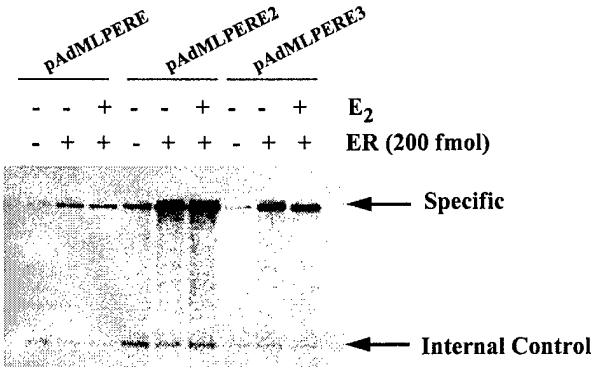

Figure 1. Stimulation of transcription *in vitro* by partially purified baculovirus expressed human ER. The transcriptional activity of ER was analyzed by *in vitro* transcription assay. Two hundred fmol of ER and 1 μM E_2 and 10 ng of the templates were used. The specific transcript (upper arrow) is from ERE-containing templates pAdMLPERE, pAdMLPERE2 or pAdMLPERE3. The internal control transcript is from pAdMLPmERE template.

Figure 1 shows that the partial purified human ER expressed in baculovirus did not affect transcription on the internal control template which contains a mutated ERE site; it did significantly increase transcription from the specific template with one to three copies of ERE sites in the absence of estradiol (E_2) (Fig. 1). However, E_2 did not further increase ER-mediated transcription (Fig. 1) as reported previously. Although the amount of HeLa nuclear extract (30 to 60 μg) did not have significant effect on ER-mediated transcription *in vitro* (data not shown), the concentration of the specific template was a critical factor on ER-mediated effect in this system as shown in Figure 2.

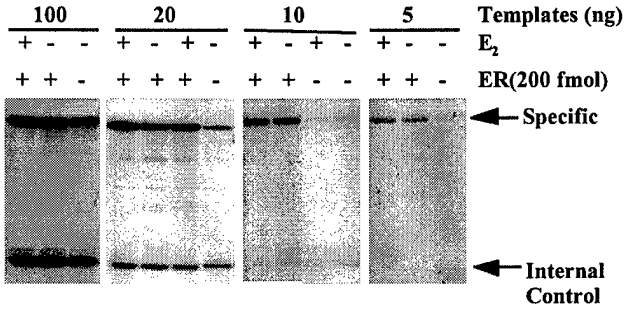

Figure 2. The concentration of the DNA template (pAdMLPERE3) had an important effect on ER-mediated transcription *in vitro*. The assay was performed as in Figure 1 except that various amounts of templates (present in ng) were used.

When a high concentration of template such as 100 ng/reaction is used, the ER did not stimulate transcription from the ERE-containing template because of the high basal activity. However, the ER significantly increased the level of the specific transcript when 20 ng/reaction or less of the template is used.

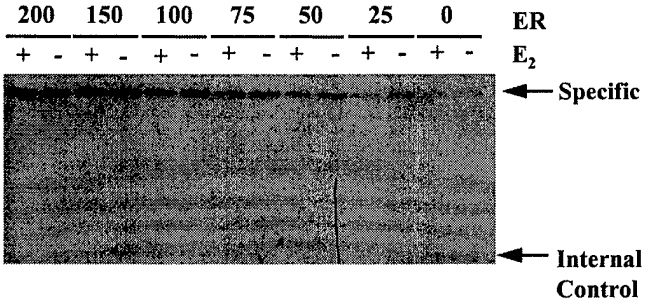

Figure 3. The concentration-dependent effect of the ER on ER-mediated transcription *in vitro*.

Figure 3 elucidated a concentration-dependent effect of the ER on ER-mediated transcription *in vitro*. With increasing concentration of the ER, ER-mediated transcription increased. However, the ER did not further stimulate transcription at the concentrations higher than 400 fmol (data not shown) and as the matter of fact the transcription level slightly decreases as shown previously.

ER activated ERE containing promoter in the absence of E_2 *in vitro*, which was different from the situation *in vivo*. There are two potential possibilities for the E_2 independence. First, using circular plasmids as the templates *in vitro* instead of chromatin templates *in vitro* rules out the requirement of estrogen to activate ER function. This issue has been addressed in a recent publication. The other possibility is that there was estrogenic activity in the cell free transcription system. The endogenous estrogenic activity in the system might activate transcriptional activity of the ER in the absence of exogenous E_2. To study hormone-dependent effect on ER-mediated transcription *in vitro*, three antiestrogens including 4-hydroxytamoxifen (4-OH TAM), ICI164,384 and ICI182,780 were tested in the system. ICI164,384 and ICI182,780 are pure ER antagonists and 4-OH TAM is a partial ER agonist and antagonist. In Figure 4, the ER stimulated transcription from the specific template independent of E_2 as shown earlier. ICI164,384 significantly inhibits ER-mediated transcription in a concentration-dependent manner. When exogenous E_2 was added, E_2 overcomes the inhibitory effect of ICI164,384 and induces ER-mediated transcription. Similar results are obtained when ICI182,780 and 4-OH TAM were used.

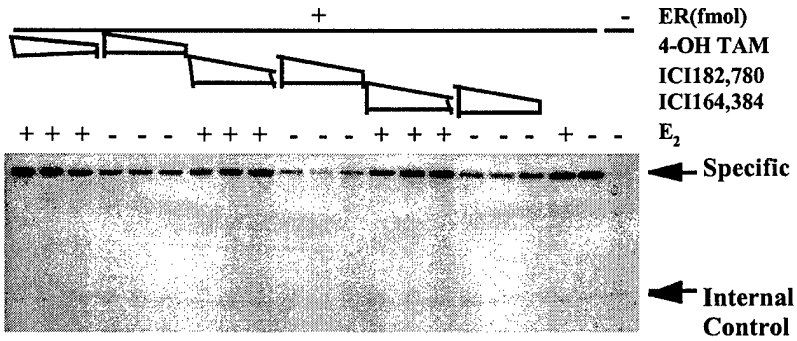

Figure 4. Antiestrogens inhibited ER-mediated transcription *in vitro*. The assay was carried out in the presence of vehicle, ICI 164,384 (4 to 400 μM), ICI182,780 (4 to 400 μM), 4-OH TAM (0.4 to 40 μM), E_2 (1 μM) or combination of ICI164,384 and E_2, combination of ICI182,780 and E_2 or combination of 4-OH TAM and E_2.

How do the antiestrogens inhibit ER-mediated transcription? We investigated the effects of the antiestrogens on ER DNA binding ability. ER-ERE complex migrated faster when ER bound to E_2 than that in the absence of hormone or in the presence of 4-OH TAM. In ERE gel mobility shift assay (Fig. 5), E_2 did not alter the migration rate of ER-ERE

complex, further suggesting that ER already bound to E_2. ICI164,384 and ICI182,780 inhibited the formation of ER-ERE complex, which might contributes to the inhibitory effects of these two antagonists on ER-mediated transcription. 4-OH TAM does not affect the formation of ER-ERE complex. However, the migration of the ER-ERE complex in the gel was slightly slower when ER binds to 4-OH TAM as seen previously, suggesting that 4-OH TAM induced a conformation change of the complex which might affect the communication of ER and basal transcription factors and resulted in inhibition of ER-mediated transcription.

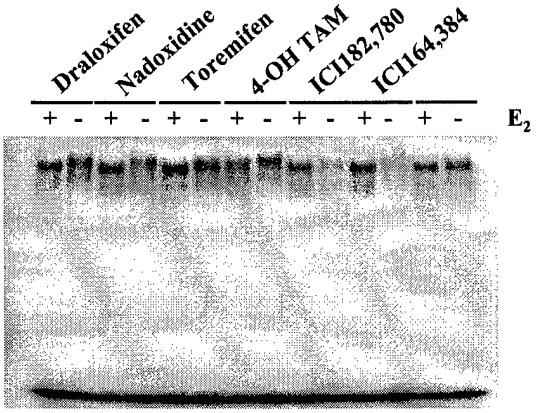

Figure 5. The effect of ligands on ER-ERE gel complex formation.

SRC-1 is cloned as a steroid hormone receptor coactivator. It might be one of the communicators between ER and the basal transcription factors. To investigate the effect of SRC-1 on ER-mediated transcription *in vitro*, SRC-1 was constructed as a glutathione-*S*-transferase (GST) fusion protein (GST-SRC-1) and expressed in bacteria. As shown in Figure 6, GST-SRC-1 slightly increased basal transcription level from the internal control template and the transcription from the specific template in the absence of ER. When added with ER, GST-SRC-1 significantly increased ER-mediated transcription in a concentration-dependent way.

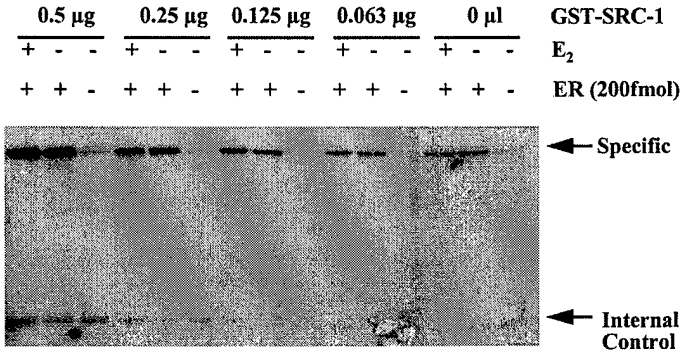

Figure 6. GST-SRC-1 significantly augmented ER-mediated transcription *in vitro*.

There are several proteins found to associate with ER hormone binding domain (HBD) in a hormone dependent manner. These proteins bind to the ER hormone binding domain in the presence of E_2 and its synthetic analog diethylstilbestrol (DES). They do not associate with ER hormone binding domain when antiestrogens such 4-OH TAM, ICI164,384 and ICI182,780 are present.

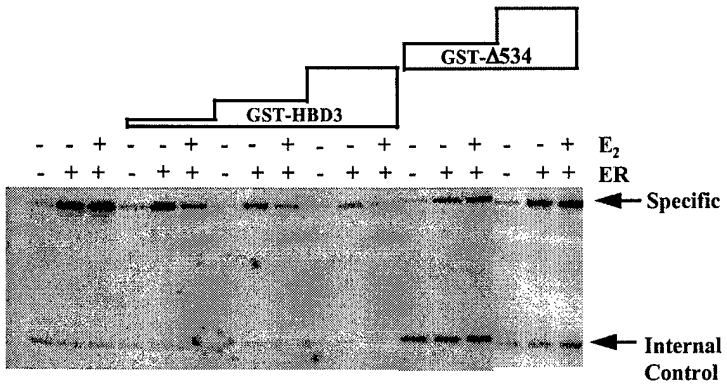

Figure 7. The ER HBD had dominant negative effect on ER-mediated transcription.

To determine the effects of these ER HBD associated proteins on ER-mediated transcription *in vitro*, we used GST-HBD3 that GST fuses to ER HBD as a dominant negative competitor for the ER. Figure 7 showed that GST-HBD3 did not affect the basal transcription level. However, it significantly inhibits ER-mediated transcription. In contrast, GST-Δ534 was used as a negative control. GST-Δ534 is lack of 535-595 amino acids of ER. It bound to hormone, but did not bind to ER associated proteins. GST-Δ534 did not affect ER-mediated transcription as shown in Figure 7.

If ER associated proteins are required for ER-mediated transcription, Hela nuclear extracts depleted of the ER associated proteins will not support ER-mediated transcription *in vitro*. To test this hypothesis, we prepared depleted Hela nuclear extracts by incubating with GST-HBD3-sepharose.

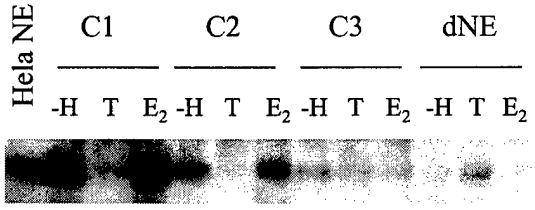

Figure 8. Far western blot. C1, C2 or C3 are the GST-HBD3-sepharose bound ER associated proteins after first, second or third depletion cycle. dNE is depleted nuclear extract.

Figure 8 shows that the ER associated proteins bound to GST-HBD3-sepharose in a hormone-dependent manner. After three-depletion cycle, an undetectable level of ERAPs was left in the depleted nuclear extract in the absence or presence of E_2 but a detectable level of ERAPs was left in the depleted nuclear extract in the presence of 4-OHT. Surprisingly, all three depleted nuclear extract (no hormone, E_2 or 4-OHT) supported ER-mediated transcription *in vitro* to the same extent as the original nuclear extracts (Fig. 9).

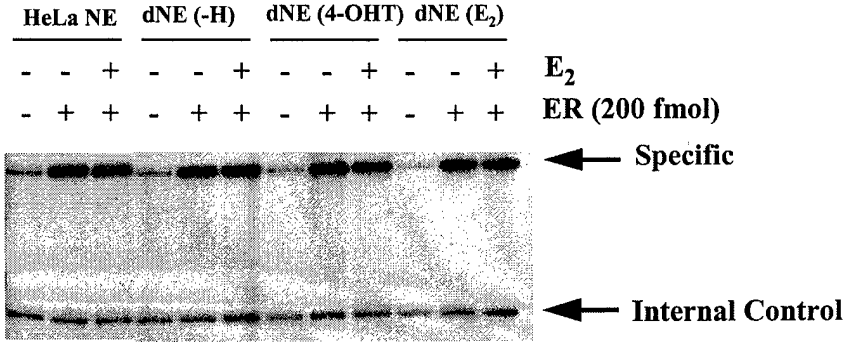

Figure 9. ER-mediated transcription *in vitro* in the depleted HeLa nuclear extracts (dNE). dNE (-

H), dNE(E_2) or dNE(4-OHT) were generated in the absence of hormones, estradiol or 4-hydroxytamoxifen, respectively.

In summary, I have completed the project outlined in the approved Statement of Work.

1. ER-mediated transcription *in vitro* is ligand-dependent; antiestrogens such as ICI164,384, ICI182,780 and 4-hydroxytamoxifen inhibited transactivation activity of the ER and estradiol induced ER-mediated transcription;
2. The hormone binding domain of the ER had dominant negative effect on ER-mediated transcription;
3. SRC-1 significantly augmented ER-mediated transcription *in vitro*.
4. Depletion of ER associated proteins from HeLa nuclear extracts did not affect ER-mediated transcription in this study.

Appendix

1) A bulleted list of key research accomplishments

I have finished the work proposed in my fellowship proposal. The research accomplishments are stated in detail as following:

- I have optimized the condition for ER-induced ERE site-dependent transcription and have showed ligand-dependent ER-mediated transcription *in vitro*: antiestrogens such as ICI164,384, ICI182,780 and 4-hydroxytamoxifen inhibited transactivation activity of the ER and estradiol induced ER-mediated transcription;
- The hormone binding domain of the ER had dominant negative effect on ER-mediated transcription;
- GST-SRC-1 significantly augmented ER-mediated transcription *in vitro*.
- Depletion assay: Prepared large amounts of Heal nuclear extracts, and deplete the ERAPs from the extracts and test the ER-mediated transcription in the depleted system. Depletion of ER associated proteins (ERAPs) from HeLa nuclear extracts did not affect ER-mediated transcription in this study. Thus, ERAPs are not essential for ER function.

2) A list of reportable outcomes

Meeting Abstracts

H. Liu and V. C. Jordan (1998). The mechanism of the estrogenic activities of estrogens and antiestrogens in MDA-MB-231 human breast cancer cells transfected with a naturally occurring mutated estrogen receptor. Xth International Congress on Hormonal Steroids, June 17-21, Quebec City.

H. Liu, R. Meyer, J. DiRenzo and M. Brown (1997) Estrogen receptor-mediated transcription *in vitro.* Era of Hope, the Department of Defense Breast Cancer Research Program Meeting, Oct. 31-Nov. 4, Washington, DC.

Manuscripts

H. Liu, E-S. Lee, A. De Los Reyes, and V. C. Jordan (1999). Allosteric Silencing and Reactivation of Activating Functions in Human Estrogen Receptor alpha by Antiestrogens (submitted to PNAS USA).

H. Liu, R. Meyer, J. DiRenzo and M. Brown (1999) Estrogen receptor-mediated transcription *in vitro*. (manuscript in preparation).

Meeting Abstracts

H. Liu and V.C. Jordan (1998). The mechanism of the estrogenic activities of estrogens and antiestrogens in MDA-MB-231 human breast cancer cells transfected with a naturally occurring mutated estrogen receptor. Xth International Congress on Hormonal Steroids, June 17-21, Quebec City.

We have identified and characterized a naturally occurring mutation in the estrogen receptor (ER) from a tamoxifen-stimulated transplantable human breast tumor line. The mutation is at amino acid 351 (Asp⇒Tyr) of hormone binding domain (HBD) of ER, referred as to Asp351Tyr ER. We have shown that Asp351Tyr ER increased estrogenic activity of tamoxifen and changed the pharmacological characteristics of raloxifene from an ER antagonist to an agonist in the term of regulation of transforming growth factor (TGF) α gene expression in MDA-MB-231 ER negative human breast cancer cells. In this study, we determined the mechanism of Asp351Tyr ER-mediated estrogenic activities of estrogens and antiestrogens. Estrodial induced the binding of a series of ER associated proteins including SRC-1 and p300 to bind to the HBD of the wild type ER and antiestrogens raloxifene and 4-hydroxytamoxifen blocked the association. Surprisingly, no proteins were found to associate with the HBD of Asp351Tyr ER in a pull down assay with either estrogen or antiestrogens present, suggesting an alternative mechanism for Asp351Tyr ER-mediated transcriptional activity.

H. Liu, R. Meyer, J. DiRenzo and M. Brown (1997) Estrogen receptor-mediated transcription *in vitro*. Era of Hope, the Department of Defense Breast Cancer Research Program Meeting, Oct. 31-Nov. 4, Washington, DC.

Estrogen receptor (ER) is ligand-activated transcription activator. The detailed mechanism of ER-mediated transcription is unclear. In this study, we demonstrated ER-mediated transcription in cell-free transcription system is ligand-dependent. Antiestrogens ICI164,384, ICI182,780 and 4-hydroxytamoxifen (4-OH TAM) significantly inhibited ER-mediated transcription. Estradiol (E_2) overcame the inhibitory effect of the antiestrogens and induced ER-mediated transcription. Under the condition used for transcription assay *in vitro*, ICI164,384 and ICI182,780 inhibited ER-ERE complex formation which might contribute to the inhibitory effect of these antiestrogens on ER-mediated transcription. 4-OH TAM changed the mobility of ER-ERE complex in the gel mobility shift assay which suggests a conformational change of the complex. Steroid receptor coactivator-1 (SRC-1) significantly augmented ER-mediated transcription *in vitro*, suggesting that SRC-1 is involved in ER signaling pathway. ER hormone binding domain (HBD) that binds to ER associated proteins (ERAPs) in a ligand-dependent manner in previously studies inhibited ER-mediated transcription *in vitro*. In contrast, truncated ER HBD (ERΔ534) lacking of 535 to 595 amino acids of ER which binds to estradiol but does not associated with ERAPs did not affect ER-mediated effect, suggesting that ERAPs are required for the full transactivation activity of ER.

Classification: Major category: Biological Science; Minor category: Molecular Pharmacology.

Title: Allosteric Silencing and Reactivation of Activating Functions in Human Estrogen Receptor alpha by Antiestrogens

Authors: Hong Liu*, Eun-Sook Lee*, Alexander De Los Reyes*, and V. Craig Jordan*†

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Abbreviations footnote: ERα, estrogen receptor alpha; LBD, ligand binding domain; 4-OHT, 4-hydroxytamoxifen; AF1 or AF2, activation function 1 or 2; GST, glutathione *S*-transferase; TGFα, transforming growth factor alpha.

ABSTRACT

The antiestrogen 4-hydroxytamoxifen (4-OHT) is known to silence activating function 2 (AF2) in the ligand binding domain (LBD) of estrogen receptor alpha (ERα) but AF1, in the A/B domain of ERα, is constitutive. In contrast, using MDA-MB-231 ERα negative human breast cancer cells stably transfected with ERα cDNA and a transforming growth factor α (TGFα) gene target *in situ*, raloxifene is a complete antiestrogen that allosterically silences AF1 and AF2. A natural mutation of Asp351Tyr ERα enhances the estrogen-like properties of raloxifene but the change in pharmacology from an antagonist to an agonist is not attributed to coactivator (SRC-1, AIB1) binding to the LBD. Construction of stable transfectants of Asp351Tyr ERα with an AF1 deletion or Asp538Ala, Glu542Ala and Asp545Ala triple mutations within helix 12 in MDA-MB-231 cells shows that estrogen-like activity of raloxifene depends on AF1 and an intact helix 12. Our results show that raloxifene differs from the 4-OHT ERα complex by silencing AF1 and AF2 in the context of the MDA-MB-231 cells. Reactivation of estrogen-like properties of raloxifene by the Asp351Tyr ERα occurs by allosteric enhancement of AF1 in the presence of an intact AF2. The activating functions of ERα can both be modulated by appropriate ligands in the LBD.

Estrogen receptor alpha (ERα), a member of the nuclear hormone receptor superfamily is a ligand-dependent transcriptional factor (1-4). Like all members in this superfamily, ERα has A to F domains from the N-terminus to the C-terminus (Table 1), containing activating function 1 (AF1) and AF2 (5-7). AF1 which is localized in the N-terminal A/B region is believed to be constitutive in a cell- and promoter-specific manner and responsible for the partial agonist activity of tamoxifen (8, 9). AF2 resides in the C-terminal ligand binding domain (LBD, region E) and has estrogen-dependent transcriptional activity by recruiting co-activators such as ERAP160/140 (10), RIP140 (11), SRC-1 (12), TIF2/GRIP1 (13-15), AIB1 (16). After binding to estrogens, ERα forms a homodimer (17) and binds via region C (DNA binding domain) to estrogen response elements in the promoter region of an estrogen responsive gene to regulate expression of genes such as transforming growth factor alpha (TGFα) (18-27).

Tamoxifen, a nonsteroidal antiestrogen (28), is the endocrine treatment of choice for all stages of breast cancer (29). Early laboratory studies (30-37) and recent clinical evidence (38) indicate that tamoxifen and its active metabolite 4-hydroxytamoxifen (4-OHT) prevent estrogen-stimulated tumor growth by competitively blocking the action of ERα. Additionally, tamoxifen maintains bone density in rats and human (39-41). However, tamoxifen is not a pure antiestrogen and the estrogen-like properties are associated with a two- to three- fold elevation in the risk of endometrial cancer (42). A related compound, raloxifene, is used clinically for the prevention of osteoporosis (43, 44) and is being tested against tamoxifen for the prevention of breast cancer in high risk women (42, 43, 45, 46). Raloxifene and its analogs are less estrogen-like than tamoxifen in the rodent uterus (47-50) and are more inhibitory than tamoxifen and 4-OHT (33) on the growth of breast cancer cells in culture (50). Recent studies demonstrate that raloxifene is virtually nonestrogenic in the human uterus

(43, 51, 52). However, there is currently no adequate molecular mechanism to explain the different intrinsic activities of the raloxifene- and 4-OHT-ERα complexes.

An important structural feature of antiestrogens is a correctly positioned alkylaminoethoxy side chain (53). Early models of antiestrogen action proposed that the side chain prevented the conformational change necessary to activate the receptor by binding to an "antiestrogenic region" (54, 55). Evidence from X-ray crystallography shows that the side chain of raloxifene interacts with aspartic acid 351 (Asp351) in the LBD. Interesting enough, an Asp351Tyr ERα mutant (56) alters the pharmacology of raloxifene from an antiestrogen to a partial agonist (57). There are at least two possible mechanisms that could explain the enhanced estrogen-like activity of the Asp351Tyr ERα raloxifene complex: either 1) helix 12 of the Asp351Tyr ERα-raloxifene complex is now repositioned to re-activate AF2 or 2) AF1 activity is enhanced allosterically. We have addressed these possibilities by stably transfecting cDNAs of mutated ERα into MDA-MB-231 human ERα negative breast cancer cells (58). Biological activities were assessed by measuring TGFα mRNA levels induced *in situ*. We have compared and contrasted raloxifene and 4-OHT to support the concept that although 4-OHT silences AF2 and reactivates AF1 activity, raloxifene actually silences both AF1 and AF2 in the context of MDA-MB-231 breast cancer cells. Asp351Tyr ERα allosterically enhances agonist activity of raloxifene without changing AF2 activity. However, the Asp351Tyr ERα-raloxifene complex becomes more estrogen-like by reactivating AF1 activity. We demonstrate, for the first time, that AF1 can be activated or silenced by an allosteric mechanism at amino acid 351.

MATERIALS AND METHODS

Plasmids Construction. pSG5HEGO, a wild type ERα expression vector, was a kind gift from Professor Pierre Chambon. pSG5HETO, an Asp351Tyr ERα expression vector, was described previously (59). To make pSG5ERα/AF2G or pSG5ERα/AF2T expression vectors, the fragments of amino acid 181 to 595 of ERα or Asp351Tyr ERα were generated from pSG5HEGO or pSG5HETO, respectively, by PCR using a 5' primer (5'-GGAATTCATGAAGGAGACTCGC-3') and a 3' primer (5'-GGAATTCTCAGACTGTGGCAGGG-3'). The PCR products were inserted into the pSG5 vector at the *EcoR* I site. pSG5ERα/AF1G and pSG5ERα/AF1T expression vectors were generated based on pSG5HEGO or pSG5HETO, respectively, using the QuickChange site-directed Mutagenesis Kit (Strategene, La Jolla, CA). The pair of primers used was:

5'-GCCCCTCTATGCCCTGCTGCTGGCGATGCTGGCCGCCCACCGCC-3'

5'-GGCGGTGGGCGGCCAGCATCGCCAGCAGCAGGGCATAGAGGGGC-3'.

The point mutations are underlined.

pGEX-HBD3, an expression vector for a glutathione *S*-transferase (GST) fusion protein containing the LBD of the human wild type ERα (GST-HBD3), was generously provided by Dr. Myles Brown (10). pGEX-HBDt, an expression vector for GST-HBDt containing the Asp351Tyr mutation, was constructed by replacing the *Hind* III/*EcoR* I fragment (778 bp) of pGEX-HBD3 with the *Hind* III/*EcoR* I fragment (778 bp) of pSG5HETO.

The sequences of all plasmids were confirmed by sequencing analysis (ABI automated sequence).

Cell Culture. MDA-MB-231 ERα negative human breast cancer cells were obtained originally from American Type Culture Collection (ATCC, Rockville, MD). MDA-MB-231, S30, BC2 and the other stable transfectants generated for this study were maintained as previously described (60).

GST Pull Down Assay. A GST pull down assay was performed as previously described (10, 61). [^{35}S]-SRC-1 and [^{35}S]-AIB1 were made from pBK-CMV-SRC-1 (kindly provided by B. O'Malley, Baylor College of Medicine, Houston, TX) and pCDNA3.1-AIB1 (kindly provided by P. Meltzer, National Institute of Health, Bethesda, MD), respectively, using an *in vitro* transcription-coupled translation system (Promega, Madison, WI).

Stable Transfection. MDA-MB-231 cells were electroporated with 10 μg of pSG5ERα/AF2G, pSG5ERα/AF1G, pSG5ERα/AF2T or pSG5ERα/AF1T expression vectors and 1 μg pBK-CMV (Strategene) to generate MDA-AF2G, MDA-AF1G, MDA-AF2T or MDA-AF1T (Table 1), respectively, as described elsewhere (59). Neomycin resistant clones were screened and characterized for ERα expression using Northern and Western blot analysis and hormone binding assays.

Western Blot Analysis. Fifty micrograms of whole cell lysates were separated on a 7.5% SDS-PAGE gel. Anti-ERα monoclonal antibody AER311 was from NeoMarker (Fremont, CA). Anti-mouse IgG conjugated with horseradish peroxidase was use to visualized bands using an ECL kit (Amersham Corp., Arlington Heights, IL).

Ligand Binding Assay. Ligand binding assays in stable transfectants were performed as previously described (62). For saturation binding assays, the stable transfectants were incubated with increasing concentrations of [^{3}H]-E_2 (Amersham Corp., Arlington Heights, IL) for 2 hr at room temperature. For competition binding assays the stable transfectants were incubated with 1 nM [^{3}H] E_2, with increasing concentrations of different ligands including 4-OHT, raloxifene or ICI182,780 for 2 hr at room temperature. To determine nonspecific binding, each concentration of [^{3}H] E_2 was competed with 400-fold excess of unlabeled diethylstilbesterol (DES). The specific binding was obtained by subtracting the nonspecific

binding from the total binding. The data were analyzed by GraphPad Prism (GraphPad Prism Software Inc.)

Northern Blot Analysis. Analysis of TGFα mRNA expression was assessed by Northern blots as previously described (63). β-Actin mRNA was detected as the loading control. The Northern blot results were quantitated using ImageQuant (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

Agonist Activities of 4-OHT-ERα Complex and 4-OHT- or Raloxifene-Asp351Tyr ERα Complexes. TGFα is one of the genes that can be upregulated by estrogens through ERα (18-27). To study the agonist activities of 4-OHT and raloxifene, we investigated the induction of TGFα mRNA levels in wild type ERα stable transfectants. In S30 cells, generated by stably transfecting wild type ERα expression vector into MDA-MB-231 ERα negative human breast cancer cells (64), E_2 induced TGFα mRNA levels in a dose-dependent manner (Fig. 1, top). 4-OHT also enhanced TGFα mRNA levels and behaved as an ERα agonist in this assay as reported previously (60, 65). Raloxifene and ICI182,780 did not increase TGFα mRNA levels and inhibited E_2-induced (Fig. 1, top) and 4-OHT-induced (data not shown) TGFα gene expression.

The estrogenic activity of E_2 on TGFα gene expression could be mediated by AF2 at the C-terminus or/and AF1 at the N-terminus of the ERα. The binding of 4-OHT inhibits binding of coactivators to the LBD of ERα (10) by promoting a distinct structural conformational change of the receptor from the E_2-ERα complex and hindering coactivator binding (66), suggesting that 4-OHT silences transcriptional activity of AF2 and the estrogenic activity of 4-OHT might be mediated through the AF1 domain (8, 9). ICI182,780 is known to compete with E_2 for the receptor, block receptor dimerization and decrease the

stability of the receptor (67, 68). The receptor levels decreased dramatically after 3 hour treatment with 1 μM ICI182,780 in our system (data not shown). This characteristic of ICI182,780 explains its inhibitory effect on the agonist activities of E_2 and 4-OHT. Raloxifene also competes with E_2 for ERα and causes a conformational change of the receptor that prevents AF2 coactivator binding (69). However, our recent study shows that raloxifene does not have a significant effect on ERα levels (58). These observations lead us to conclude that raloxifene inhibits transcriptional activity by silencing both AF1 and AF2 of ERα in S30 cells.

We also determined the agonist activities of 4-OHT and raloxifene through Asp351Tyr ERα. In BC2 cells, both E_2 and 4-OHT increased TGFα mRNA levels and the magnitude of induction was higher than that in S30 cells. More interestingly, raloxifene became estrogenic (Fig. 1, bottom). These observations are consistent with previous publications (57, 60, 63).

According to the crystallographic structure of the receptor, Asp351 forms a salt bridge or a hydrogen bond with the side chain of 4-OHT (66) or raloxifene (69), respectively, which, in turn, is believed to be important for the repositioning of helix 12 to prevent binding of coactivators. However, the question to be addressed is whether helix 12 can be repositioned in raloxifene- or 4-OHT-Asp351Tyr ERα complex to allow the binding of coactivators. In other words, can Asp351Tyr ERα reactivate AF2 by raloxifene or 4-OHT?

AF2 Silencing by 4-OHT and raloxifene. Steroid receptor coactivators (SRCs) are a family of homologous proteins including SRC-1 (12), GRIP1/TIF2 (13, 70) and p/CIP/AIB1/RAC3/ACTR (16, 71-73). They bind to LBDs of nuclear receptors and augment nuclear receptor-mediated transactivation activities in a ligand-dependent manner (10, 12-16, 70-73). Coactivator binding to the LBD of ERα correlates well with AF2 activation (10, 12, 74). To address the possible reactivation of AF2 by 4-OHT or raloxifene, we conducted GST pull down assays using GST-HBD3 and GST-HBDt to determine the interaction of coactivators with the LBDs of ERα or Asp351Tyr ERα. GST-HBDt and GST-HBD3 had

similar binding affinities for E_2, 4-OHT, raloxifene and ICI182,780, using standard competition binding assays (Data not shown).

[^{35}S]-AIB1 only bound to GST-HBD3 or GST-HBDt in the presence of E_2. Antiestrogens did not induce the association of [^{35}S]-AIB1 with GST-HBD3 or GST-HBDt (Fig. 2A). Raloxifene (shown), 4-OHT and ICI 182,780 (not shown) inhibited E_2-induced binding of [^{35}S]-AIB1 to GST-HBD3 and GST-HBDt. Similar results were obtained when [^{35}S]-SRC-1 was used (data not shown). When [^{35}S]-metabolically labeled whole cell extracts from MDA-MB-231 (Fig. 2B) and MCF-7 cells (data not shown) were used, several proteins (266, 157, 124, 105 and 32.6 kDa estimated from SDS-PAGE) were bound to GST-HBD3 in the presence of E_2 but not in the presence of antiestrogens as previously described (10, 75). Surprisingly, there were no proteins bound to GST-HBDt specifically in the presence of any tested ligands including E_2. It is possible that the Tyr351 in GST-HBDt may be modified in some way (such as phosphorylation by tyrosine kinases) in whole cell lysates (containing phosphatase inhibitors) and cause repositioning of helix 12 to prevent coactivator binding to the GST-HBDt in the presence of E_2. However, phosphorylation and dephosphorylation are in equilibrium *in vivo*. So that Asp351Tyr ERα could be partially dephosphorylated *in vivo* by phosphatases and bind to coactivators in the presence of E_2 and AF2 could be partially activated.

These results showed that 4-OHT and raloxifene did not induce AF2 coactivator binding to LBDs of ERα or Asp351Tyr ERα, suggesting that 4-OHT and raloxifene do not reactivate the AF2 domain. The next question we addressed was what roles AF1 and AF2 of ERα and Asp351Tyr ERα play in transcriptional activation of TGFα.

Stable Expression of Different ERα Mutants in MDA-MB-231 ERα Negative Human Breast Cancer Cells. To study the roles that AF1 and AF2 play in agonist activities of 4-OHT-and raloxifene-ERα or Asp351Tyr ERα complexes, we stably transfected

ERα/AF2G, ERα/AF1G, ERα/AF2T or ERα/AF1T cDNAs (Table 1) into MDA-MB-231 cells to generate MDA-AF2G, MDA-AF1G, MDA-AF2T or MDA-AF1T, respectively. Biological activity was evaluated for different ligands at the TGFα gene *in situ*. The ERα mutants used in this study were expressed to similar levels by Western blot analysis (Fig. 3). ERα/AF1G and ERα/AF1T which failed to bind to SRC-1 *in vitro* (data not shown) migrated slightly faster than the receptors in S30 and BC2, most likely due to the three charged amino acids (Asp538, Glu542 and Asp545) that are mutated to alanine. ERα/AF1G and ERα/AF2G had similar binding affinities as wild type ERα for E_2, 4-OHT, raloxifene and ICI 182,780 (Table 1). Asp351Tyr ERα had lower affinities for E_2, 4-OHT, raloxifene and ICI 182,780 compared with wild type ERα. ERα/AF1T and ERα/AF2T had similar ligand binding activities for the tested ligands as Asp351Tyr ERα except that ERα/AF1T has a relatively lower affinity for raloxifene (Table 1).

E_2 induced TGFα mRNA at a concentration as low as 10^{-12} M (1.37 times over control) and maximal induction was reached at 10^{-9} M (4.36 times) in S30 cells (Fig. 1, top). Although ERα/AF2G with an intact AF2 domain has a similar binding affinity to E_2 (Table 1), it did not enhance TGFα mRNA until the E_2 concentration was as high as 10^{-9} M and the maximal induction was 3.19 times at 10^{-8} M (Fig. 4, MDA-AF2G). The transcriptional activity of ERα/AF2G is believed to be attributed to the AF2 domain. However, ERα/AF1G, that did not bind to SRC-1 *in vitro*, induced a TGFα mRNA at a lower concentration (10^{-10}M) compared to ERα/AF2G and to a slightly higher extent (4.5 times) at 10^{-8} M (Fig. 4, MDA-AF1G). These data indicated that AF1 and AF2 can induce TGFα mRNA independently in the presence of E_2, and AF1 has stronger transcriptional activity than AF2 in MDA-MB-231 cells.

A single point mutation at amino acid 351 from an aspartic acid to a tyrosine results in lower binding affinities for ligands including E_2, 4-OHT, raloxifene and ICI 182,780 (Table 1). However, the estrogenic activities of E_2 and 4-OHT in terms of induction of TGFα mRNA are

enhanced, and raloxifene is estrogenic (Fig. 1, bottom) (57, 60, 63). Our pull down data (Fig. 2) showed that the Asp351Tyr point mutation did not enhance the mutant receptor to bind to coactivators, suggesting that AF2 is not activated. The question is whether AF1 alone or a combination of AF1 and an intact AF2 contribute to the estrogenic activity of Asp351Tyr ERα. Since neither AF1 nor AF2 alone supported the estrogenicity of 4-OHT in MDA-AF1G or MDA-AF2G cells (Fig. 4), we did not expect that 4-OHT and raloxifene would have estrogenic activity in MDA-AF1T or MDA-AF2T cells. The results in Fig. 4 confirmed our prediction. However, E_2 only slightly induced TGFα mRNA at 10^{-8}M concentration in MDA-AF2T cells. This slight activation of AF2 in MDA-AF2T cells might be due to a small portion of the receptor that is dephosphorylated and bound to coactivators as discussed earlier. Surprisingly, E_2 had no estrogenic activity in MDA-AF1T cells. These results indicate that E_2 behaves more like 4-OHT or raloxifene in these Asp351Tyr ERα containing cells. Both AF1 and an intact AF2 are needed for induction of TGFα mRNA levels by Asp351Tyr ERα in the presence of E_2, 4-OHT or raloxifene.

It is believed that agonist activity of 4-OHT is due to transcriptional activity of AF1 in a cell- and promoter-dependent manner (8, 9). 4-OHT induced TGFα gene expression in S30 cells (Fig. 1) without inducing coactivator binding to GST-HBD3 (Fig. 2), suggesting that 4-OHT could activate TGFα gene expression through the AF1 domain. It is not surprising that 4-OHT did not induce TGFα mRNA through ERα/AF2G which does not contain the AF1 domain (Fig. 4, MDA-AF2G). However, 4-OHT also failed to enhance TGFα mRNA in ERα/AF1G containing cells (Fig. 4, MDA-AF1G), suggesting that the agonist activity of 4-OHT in these cells is different from that of E_2, which induced TGFα mRNA level through ERα/AFG1. It has been reported previously that the 4-OHT-ERα complex has lower estrogenic activity in different cell contexts when AF2 is mutated (76). However, it is important to point out that the 4-OHT-ERα/AF1G complex had no agonist activity in our

system at all. Moreover, 4-OHT inhibited E_2-induced TGFα mRNA and behaved as an antagonist in MDA-AF1G cells. These results demonstrate that intact AF2 and AF1 domains of ERα are required for TGFα gene expression in the presence of 4-OHT. Raloxifene, which is different from 4-OHT, did not induce TGFα mRNA at all in S30 cells (Fig. 1, top). Although crystal structures of the LBD of ERα with 4-OHT and raloxifene (66, 69) are very similar, there are significant differences in the two structures, especially the interaction properties and the distance between the side chains of 4-OHT and raloxifene and Asp351, and the position of helix 12. The positions of the side chains of 4-OHT and raloxifene affect repositioning of the hinge chain between helix 11 and 12 and ultimately affect the positions of helix 12. 4-OHT binding produces a properly positioned helix 12 that sits in the same hydrophobic groove as the nuclear receptor box II peptide (66). In turn the repositioned helix 12 alone or in combination with other domains such as AF1, may form a surface for general transcriptional factors or unmask the inhibitory effect on AF1 to induce TGFα mRNA. The repositioning of helix 12 induced by raloxifene, which is slightly different from that by 4-OHT, can not induce the same conformational changes. This may explain why raloxifene has no agonist activity in S30 cells.

The crystallographic structures of the LBD occupied by estrogens or antiestrogens have provided valuable insight into understanding the mechanism of ERα actions (66, 69). When estrogens are present, the hinge chain (LysCysLysAsnValValPro) between helix 11 and helix 12 is closer to helix 3, and helix 12 is positioned over the ligand binding pocket. In contrast, the side chains of 4-OHT and raloxifene stick out between the hinge and helix 3 and results in the repositioning of helix 12 to prevent coactivators from binding. However, the single point mutation to replace an aspartic acid for a tyrosine at amino acid 351 may change the relationship between the hinge and helix 3 because tyrosine has a bulky side chain. Modification of the side chain, such as phosphorylation and acetylation will make it even

bulkier. Therefore, Asp351Tyr could disrupt the hinge between helix 11 and helix 3 further out of position, even in the presence of E_2. This would explain why E_2 does not induce coactivator binding and behaves like 4-OHT or raloxifene in MDA-AF1T and MDA-AF2T cells. Studies are currently underway to elucidate the role of amino acid 351 in the allosteric silencing and reactivation of AF1 in antiestrogen ERα complexes.

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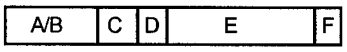
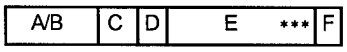
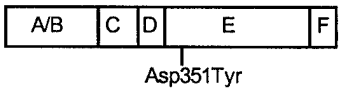
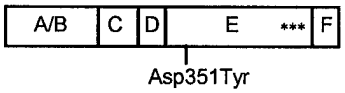
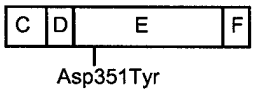
FIG.1. Northern blot analysis of TGFα mRNA in S30 (wild type ERα, top) and BC2 (Asp351Tyr ERα, bottom) cells. The cells were treated with 10^{-12} to 10^{-8} M of E_2, 10^{-6} M of 4-OHT, raloxifene (Ral) or ICI 182,780 (ICI) or combination of 10^{-8} M E_2 and 10^{-6} M of indicated antiestrogens for 24 hours. Fifteen micrograms of total RNA were loaded in each lane. β-Actin was used as the loading control.

FIG.2. GST pull down assays using GST-HBD3 and GST-HBDt. A) [^{35}S]-AIB was incubated with GST, GST-HBD3 or GST-HBDt in the presence of no ligand (-), 10 nM E_2 (E), 1 μM 4-OHT (T), 1 μM raloxifene (R), 1 μM ICI 182,780 (I) or combination of 10 nM E_2 and 1 μM raloxifene (R+E). B) [^{35}S]-metabolically labeled whole cell lysates of MDA-MB-231 cells were incubated with GST, GST-HBD3 or GST-HBDt in the presence of no ligand (-), 10 nM E_2 (E), 1 μM 4-OHT (T) or 1 μM raloxifene (R). Molecular weight markers are labeled on the right.

FIG.3. Western blot analysis of expression levels of mutated ERα in the stable transfectants.

FIG.4. TGFα mRNA levels in the different ERα stable transfectants by Northern blot analysis. The treatments were the same as that in Fig. 1.

Table 1. Ligand binding characteristics of mutant ERα stable transfectants

ERα Mutants		Transfectants	Kd (nM)	IC50 (nM)		
				4-OHT	Ral	ICI
Wild type ERα		S30	0.41	0.98	1.27	1.33
ERα/AF1G		MDA-AF1G	0.42	1.94	1.75	3.67
ERα/AF2G		MDA-AF2G	0.59	2.58	3.66	3.02
Asp351Tyr ERα		BC2	1.92	4.30	11.58	7.91
ERα/AF1T		MDA-AF1T	1.21	3.97	5.20	5.67
ERα/AF2T		MDA-AF2T	1.53	3.57	10.80	5.05

*** Asp538Ala/Glu542Ala/Asp545Ala triple mutations at helix 12

Fig. 1

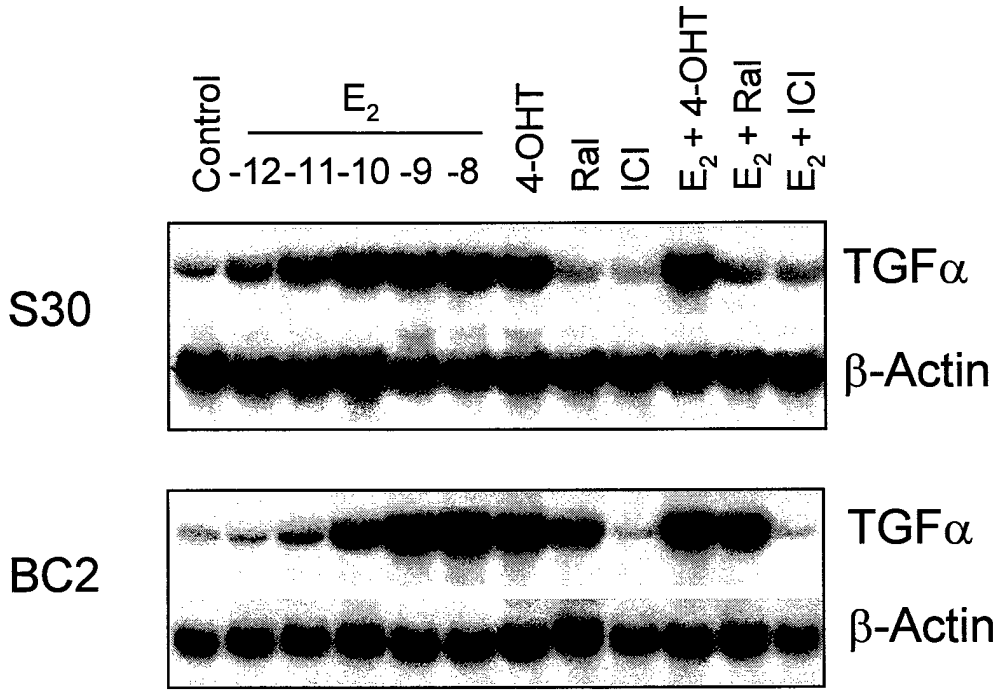

Control
E2
-12
-11
-10
-9
-8
4-OHT
Ral
ICI
E2 + 4-OHT
E2 + Ral
E2 + ICI
S30
TGFα
β-Actin
BC2
TGFα
β-Actin

Fig. 2a

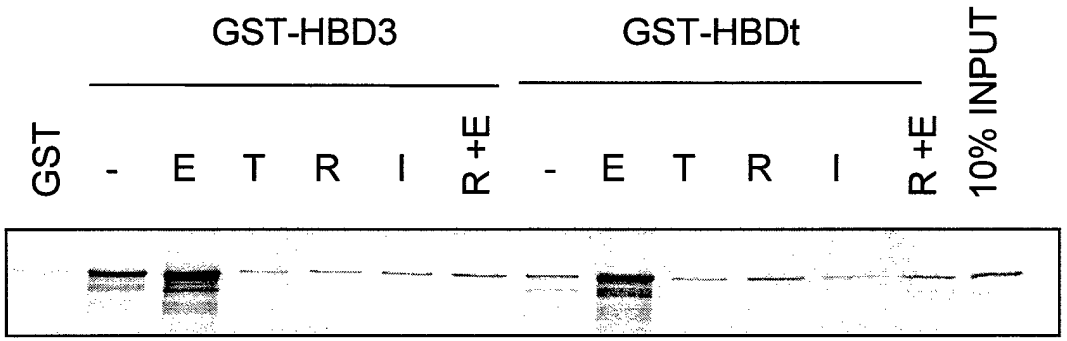


Fig. 2b

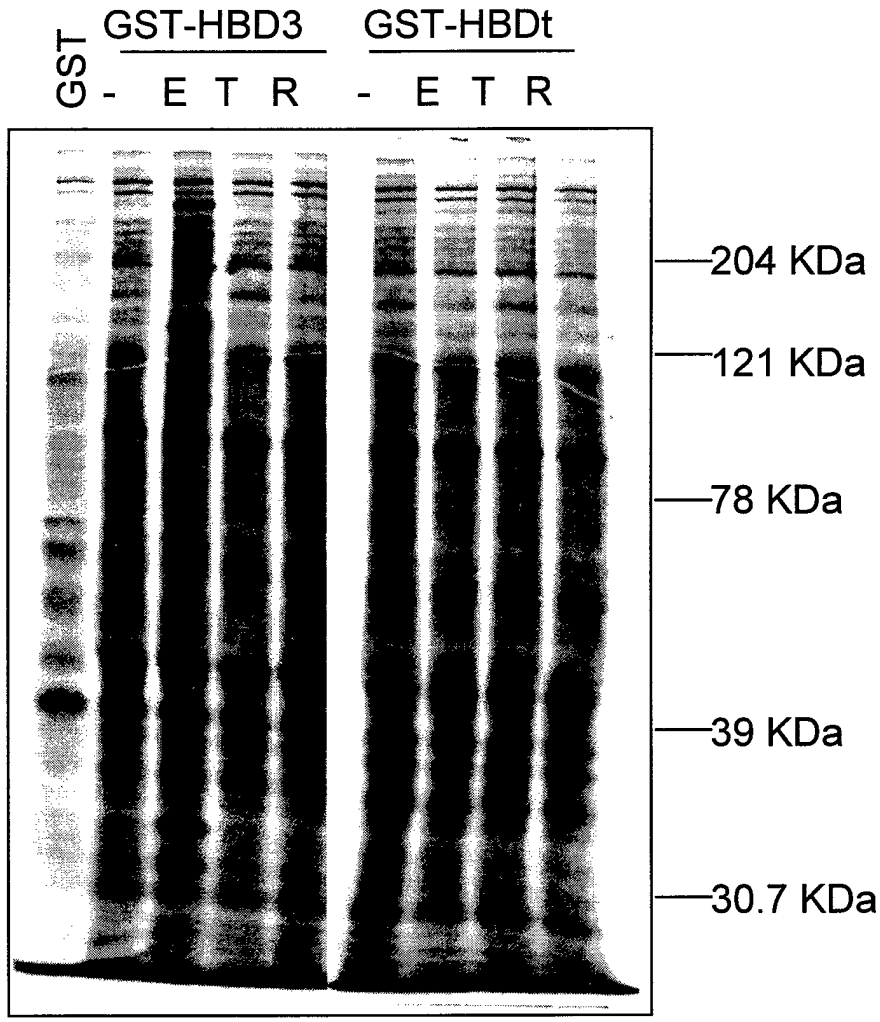

GST
GST-HBD3
GST-HBDt
- E T R - E T R
204 KDa
121 KDa
78 KDa
39 KDa
30.7 KDa

Fig. 3

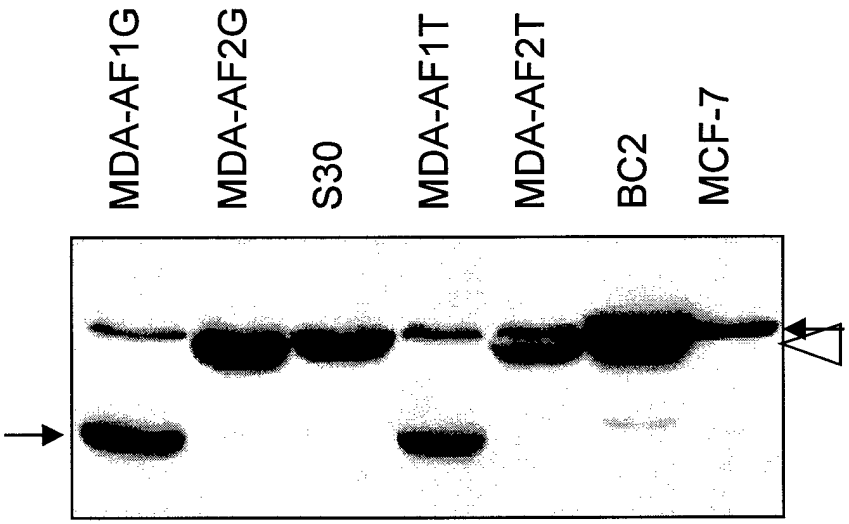
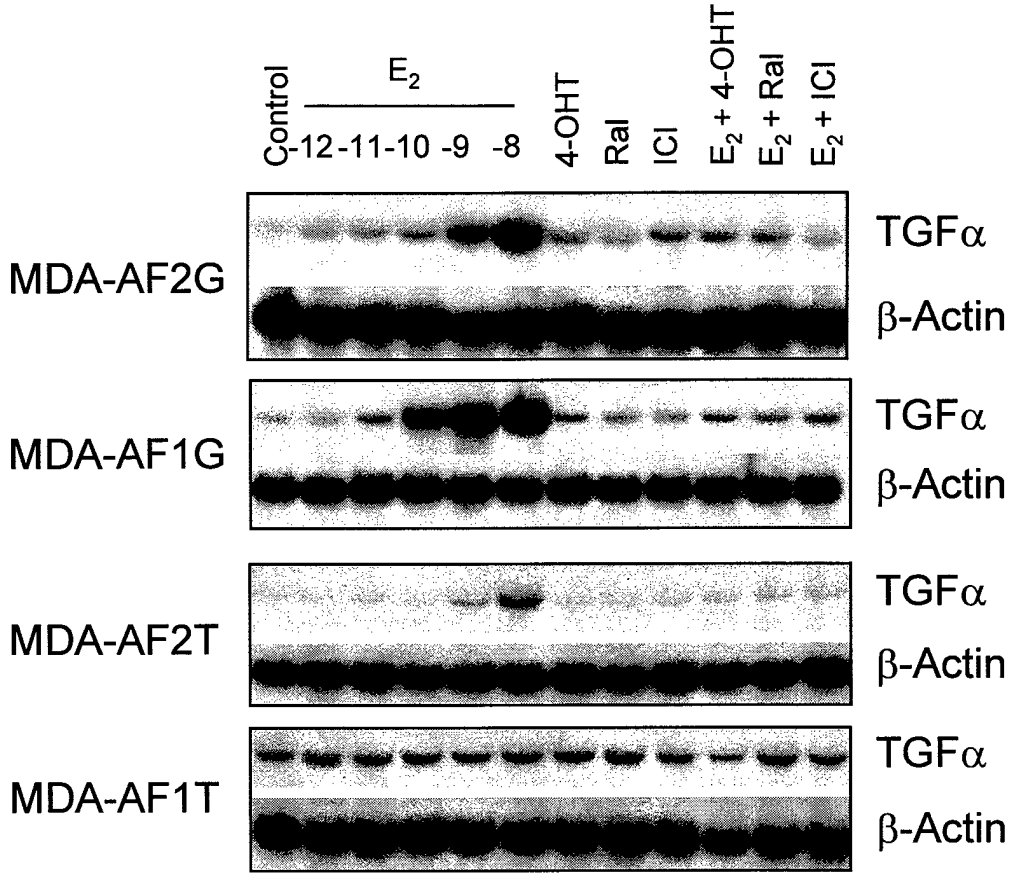


Fig. 4


Control
E2
-12 -11 -10 -9 -8
4-OHT
Ral
ICI
E2 + 4-OHT
E2 + Ral
E2 + ICI
MDA-AF2G
MDA-AF1G
MDA-AF2T
MDA-AF1T
TGFα
β-Actin

Estrogen Receptor-Mediated Transcription *in Vitro* [1]

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Running title: ER-mediated transcription *in vitro*

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[2] The abbreciations used are: ER, estrogen receptor alpha; DBD, DNA binding domain; AF, activation function; ERE, estrogen response element; ERAPs, estrogen receptor associated proteins, SRC-1, steroid receptor coactivator-1; HBD, hormone binding domain, GST, glutathione-*S*-transferase.

Estrogen receptor alpha (ER) is a ligand-activated transcription activator. To elucidate the mechanism of ER-mediated transcription in detail, we studied transcriptional activity of the ER *in* vitro. We demonstrated ER-mediated transcription in a cell-free transcription system was ligand-dependent. Antiestrogens ICI164,384, ICI182,780 and 4-hydroxytamoxifen significantly inhibited transcriptional activity of the ER. Estradiol overcame the inhibitory effect of the antiestrogens and induced ER-mediated transcription. Under the condition used for transcription assays, ICI164,384 and ICI182,780 inhibited ER-ERE complex formation which might contribute to the inhibitory effect of these antiestrogens on ER-mediated transcription. 4-hydroxytamoxifen changed the mobility of ER-ERE complex in the gel mobility shift assay, suggesting a conformational change of the complex. Steroid receptor coactivator 1 (SRC-1) significantly augmented ER-mediated-transcription *in vitro*. The hormone binding domain (HBD) of the ER that binds to estrogen receptor associated proteins (ERAPs) in a ligand-dependent manner inhibited ER-mediated transcription *in vitro*. In contrast, the truncated ER HBD (ER□534) lacking of 535 to 595 amino acids of the ER binds to estradiol but does not associate with ERAPs. ER□534 did not affect ER-mediated effect in this study, suggesting that binding to ERAPs is required for the maximal transactivation activity of the ER. However, depleting ERAPs from the cell free transcription system did not decrease ER-mediated transcription, suggesting that ERAPs are not required for the basic transcriptional activity of the ER.

Estrogen receptor (ER) is a member of nuclear hormone receptor superfamily which includes steroid hormone receptors, thyroid and retinoid hormone receptors, vitamin D receptor, and a large number of so-called orphan receptors for which no ligands have been

identified (1-4). These receptors function as ligand-activated transcription factors. The ER was identified in 1960s and the function of the ER as a transcription regulator was also proposed (5,6). Since then, extensive studies have been conducted to probe the detail mechanism of ER-mediated effects at molecular levels. With the cloning of the ER gene (7,8), significant progresses have been made in the elucidation of the structure of the ER and the dissection of the mechanism of ER-mediated signal transduction. Human ER is a protein of 595 amino acids and has a molecular weight of approximately 67 kDa (7,8). Like all members of the nuclear hormone receptor superfamily, it has A to F domains from N-terminus to C-terminus and has a structure which includes a C-terminal domain with hormone binding, dimerization and hormone-dependent activation function 2 (AF2), a hinge region, a highly conserved central DNA binding domain (DBD) with two zinc fingers, and an N-terminal domain which has autonomous transcription activation activity (AF1) in a cell- and promoter-specific manner. Although different mechanisms have been proposed, most studies on ER-mediated signal transduction have been carried out on the classic ER-ERE pathway in which the ER binds to estrogen, forms a homodimer, recognizes and binds to a palindromic cognate estrogen receptor responsive elements (EREs) with a consensus sequence of GGTCANNNTGACC which locates in the regulatory regions of ER targeted genes and then regulates gene transcription (9-14).

However, the detailed mechanism of ER-mediated transcription is still unknown. There is evidence that ER facilitates the formation of the initiation complex (15). The extensive studies on ER-mediated signal transduction led to the discovery of a set of ER associated proteins (ERAPs) which gives a much more sophisticate view of the mechanism of nuclear receptor action. Up to now, several nuclear receptor associated proteins have been identified or cloned in different laboratories, including estrogen receptor associated protein (ERAP) 160/140 (16), SRC-1 (17), co-integrator-associated protein (p/CIP)

(18)/amplified in breast cancer-1(AIB1) (19)/RAC3 (20)/ACTR (21), receptor interacting protein (RIP)140 (22), p300/CBP (23-26), transcriptional mediators or intermediary factors (TIF)1 (27), TIF2 (28)/glucocorticoid receptor interacting protein (GRIP)1 (29-31), and thyroid hormone receptor interacting protein 1 (TRIP1) (32). These nuclear receptor associated proteins bound to nuclear receptors in ligand-dependent manners correlates to the ligand-dependent transcription of the receptors in cells, suggesting a putative role of the nuclear receptor associated proteins in ligand-activated receptor-mediated transcription.

In this study, a cell-free transcription system was used to study the effects of ER-associated proteins on ER-mediated transcription. We showed that ER-mediated transcription *in vitro* was ligand-dependent under the condition used in this study. ER hormone binding domain associated proteins were required for the full transcription activity of ER. GST-SRC-1 enhanced ER-mediated transcription *in vitro*.

EXPERIMENTAL PROCEDURES

Plasmids—pAdMLPERE3 is described previously (33) (kindly provided by Dr. C. Abbondanza), which contains three copies of ERE linked to a minimal adenovirus major late promoter (-53 to +9) and a 400 nucleotides of G-less cassette as the reporter. The internal control (pAdMLPmERE) which has the same promoter and a shorter G-less cassette (200 nucleotides) was generated by replacing the three copies of ERE by a mutated ERE (5'-AGGACACAGTGTCCT-3') which abolishes the formation of ER-ERE complex (34). GST-HBD3, GST-□534 are described previously (16). hSRC-1 cDNA that is the equivalent of that reported by Onate et al (17) was subcloned as GST-SRC-1 and expressed in E. Coli strain Y1090 strain.

Protein Expression—Human ER is overexpressed in baculovirus system and partially purified by Mono S column (PanVera Inc., Madison, WI). GST-HBD3, GST-□534 and GST-SRC-1 are expressed in bacteria and purified on glutathione sepharose (Pharmcia, Poscataway, NJ).

In Vitro Transcription Assay—HeLa cells were grown in suspension and nuclear extract was prepared as previously described (35). The *in vitro* transcription reaction contained: 10 mM HEPES (pH 7.9), 8.5% glycerol, 60 mM KCl, 7.5 mM $MgSO_4$, 5 mM creatine phosphate, 2.5 mM DTT, 30 U RNasin inhibitor, 5 mg/ml BSA, 12.5 mM ATP and UTP, 5 μM CTP, 40 μM 3-O'-methy-GTP, 20 μCi [α-^{32}P]CTP (800 Ci/mmol), 10 U RNase T1. Different concentration of templates (pAdMLPERE3 and pAdMLPmERE) were used. The ER was incubated with hormones at 30°C for 20 minutes, then incubated with HeLa nuclear extract (30 - 40 μg) at 30°C for another 20 minutes followed by 30 minute incubation with templates at 30°C. The transcription was initiated by adding NTPs. Reactions were stopped by addition of 200 μl of stopping buffer (10 mM Tris (pH 7.9), 10 mM EDTA, 1 M ammonium acetate, 0.5% SDS, and 70 μg/ml yeast tRNA). The RNA was

extracted once with phenol/chloroform/isoamyl alcohol (25/24/1/) and once with chloroform/isoamyl alcohol (24/1), precipitated with 100% ethanol and separated on a 6% sequencing gel. The gel was autoradiographed at -70°C with intensifying screens.

Gel Shift Assay—ERE oligonucleotides were annealed and labeled with [α-^{32}P]dGTP (3000 Ci/mmol) by Klenow fragment. The ER was incubated under the same condition as that for *in vitro* transcription assay except that ^{32}P-ERE was used instead of plasmid templates. The ER-ERE complex was separated on a 4% polyacrylamide gel in 0.5 X TGE (34).

RESULTS

Baculovirus-Expressed Human ER Induces Transcription in Vitro—To study the function of the ER *in vitro*, a cell free transcription system was set up as described. Figure 1 shows that the partial purified human ER expressed in baculovirus did not affect transcription on the internal control template which contains a mutated ERE site; it did significantly increase transcription from the specific template with three copies of ERE sites in the absence of 1 μM estradiol (E_2). However, E_2 did not further increase ER-mediated transcription as reported previously (15,33). Although the amount of HeLa nuclear extract (30 to 60 μg) did not have significant effect on ER-mediated transcription *in vitro* (data not shown), the concentration of the specific template was a critical factor on ER-mediated effect in this system as shown in figure 2. When a high concentration of template such as 100 ng/reaction was used, the ER did not stimulate transcription from the ERE-containing template because of the high basal activity. However, the ER significantly increased the level of the specific transcript when 20 ng/reaction or less of the template was used. Figure 3 elucidated a concentration-dependent effect of the ER on ER-mediated transcription *in vitro*. With increasing concentration of the ER, ER-mediated transcription increased. However, the ER did not further stimulate transcription at the concentrations higher than 400 fmol (data not shown), and as the matter of fact the transcription level slightly decreased as shown previously (15).

ER-Mediated Transcription in Vitro is Ligand-Dependent—ER activated ERE containing promoter in the absence of E_2 *in vitro* which was different from the situation *in vivo*. There are two potential possibilities for ER-mediated transcription *in vitro* independent of E_2. First, using circular plasmids as the templates *in vitro* instead of chromatin templates *in vitro* might minimize the requirement of estrogen to activate ER function. This issue has

been addressed in a recent publication (). The other possibility is that there was estrogenic activity in the cell free transcription system. The endogenous estrogenic activity in the system might activate transcriptional activity of the ER in the absence of exogenous E_2. To study hormone-dependent effect on ER-mediated transcription *in vitro*, three antiestrogens including 4-hydroxytamoxifen (4-OH TAM), ICI164,384 and ICI182,780 were used to minimize the endogenous estrogenic activity in the system. ICI164,384 and ICI182,780 are pure ER antagonists and 4-OH TAM is a partial ER agonist and antagonist (36,37). The ER was incubated with different concentrations (4 to 400 μM) of antiestrogens without or with 1 μM E_2 in the transcription assays. In figure 4, the ER stimulated transcription from the specific template independent of E_2 as shown earlier. Pure antiestrogens, ICI164,384 and ICI182,780 significantly inhibited ER-mediated transcription. When exogenous E_2 was added, E_2 overcame the inhibitory effect of ICI164,384 and induced ER-mediated transcription. Similar results were obtained when 4-OH TAM was used.

How did the antiestrogens inhibit ER-mediated transcription? We investigated the effects of the antiestrogens on ER DNA binding ability. ER-ERE complex migrated faster when ER bound to E_2 than that in the absence of hormone or in the presence of 4-OH TAM (34). In ERE gel mobility shift assay (Fig. 5), E_2 did not alter the migration rate of ER-ERE complex (lane 1 and 2), further suggesting that ER already bound to E_2. ICI164,384 and ICI182,780 inhibited the formation of ER-ERE complex, which might contributes to the inhibitory effects of these two antagonists on ER-mediated transcription. 4-OH TAM did not affect the formation of ER-ERE complex. However, the migration of the ER-ERE complex in the gel was slightly slower when ER binds to 4-OH TAM as seen previously (34), suggesting that 4-OH TAM induced a conformation change of the complex which might affect the communication of ER and basal transcription factors and resulted in inhibition of ER-mediated transcription.

SRC-1 Is Involved in ER-Mediated Transcription in Vitro—SRC-1 is cloned as a steroid hormone receptor coactivator (17). It might be one of the communicators between ER and the basal transcription factors. To investigate the effect of SRC-1 on ER-mediated transcription *in vitro*, SRC-1 was constructed as a glutathione-*S*-transferase (GST) fusion protein (GST-SRC-1) and expressed in bacteria (see EXPERIMENTAL PROCEDURES). As shown in figure 6, GST-SRC-1 slightly increased basal transcription level from the internal control template and the transcription from the specific template in the absence of the ER. When added with the ER, GST-SRC-1 significantly increased ER-mediated transcription in a concentration-dependent way. This result indicated that SRC-1 was involved in ER-mediated transcription *in vitro*.

The Proteins Which Associate with ER Hormone Binding Domain Are Required for the Full Transcription Activation Activity of the ER—There are several proteins found to associate with ER hormone binding domain (HBD) in a hormone dependent manner (16,17,22,24). These proteins bind to the ER HBD in the presence of E_2 and its synthetic analog diethylstilbestrol (DES). They do not associate with ER hormone binding domain when antiestrogens such 4-OH TAM, ICI164,384 and ICI182,780 are present. To determine the effects of these ER HBD associated proteins on ER-mediated transcription *in vitro*, we used GST-HBD3 that GST fuses to ER HBD (16) as a dominant negative competitor for the ER. Figure 7 showed that GST-HBD3 did not affect the basal transcription level, however, it significantly inhibits ER-mediated transcription. In contrast, GST-Δ534 (16) was used as a negative control. GST-Δ534 lacks of 535-595 amino acids of ER. It bound to hormone, but did not bind to ER associated proteins (16). GST-Δ534 did not affect ER-mediated transcription as shown in figure 7.

If ER associated proteins are absolutely required for ER-mediated transcription, Hela nuclear extracts depleted of the ER associated proteins will not support ER-mediated

transcription *in vitro*. To test this hypothesis, we prepared depleted Hela nuclear extracts by incubating with GST-HBD3-sepharose (see Experimental Procedures). Figure 8 shows that the ER associated proteins bound to GST-HBD3-sepharose and after three depletion cycle, an undetectable level of the associated proteins left in the depleted nuclear extract in the absence or presence of E_2 but quite high level in the depleted nuclear extract in the presence of 4-OHT. Surpassingly, all three depleted nuclear extract (no hormone, E_2 or 4-OHT) supported ER-mediated transcription *in vitro* to the same extent as the original nuclear extracts (Fig. 9).

DISCUSSION

ER Induces Transcription in Vitro—It has been reported that ER induces transcription *in vitro* using purified calf uterus ER (33) and baculovirus expressed ER (15). In both studies, ER-mediated transcription was hormone-independent. Under certain salt and temperature condition which affects ER-ERE complex formation, ER-mediated transcription *in vitro* was estradiol-stimulated (38). The results shown in figure 1 to figure 3 also indicate that ER-mediated transcription *in vitro* is E_2-independent under the condition used. E_2-independent ER-mediated transcription *in vitro* does not correlate with the *in vivo* data that ER is a ligand-dependent transcription factor, suggesting that the ER is somehow activated during the purification procedure or there are estrogen-like activities in the ER fraction or HeLa nuclear extract, since the Sf9 cells used for expressing human ER and HeLa cells were not grown in hormone-free condition. By using antiestrogens we demonstrated that partially purified baculovirus-expressed human ER induces transcription *in vitro* in a hormone-dependent manner. In the absence of antiestrogens (Fig. 4, lane 2 and 3), ER-mediated transcription is independent of E_2 as reported previously. However, Antiestrogens ICI164,384, ICI182,780 and 4-OH TAM inhibited ER-mediated transcription (Fig. 4). According to previous study (34), E_2 speeds the migration of ER-ERE complex in ERE mobility shift assay. In figure 5 (Lane 1 and 2), E_2 did not alter the ER-ERE complex which suggests that the ER pre-bound to estrogen, explaining why ER-mediated transcription is E_2-independent in the absence of antiestrogens. ICI164,384 and ICI182,780 which are pure antiestrogens inhibit the formation of ER-ERE complex and inhibit ER-mediated transcription. Partial ER antagonist 4-OH TAM changes the migration of the ER-ERE complex and inhibits ER-mediated transcription to less extend. E_2 overcomes the inhibitory effects of the antiestrogens and induces ER-mediated

transcription.

SRC-1 is Involved in ER-Mediated Transcription in vitro—SRC-1 has been reported as a steroid hormone receptor coactivator (17). It has been demonstrated that SRC-1 augment steroid receptor-mediated transcription *in vivo*(17,39-41). The recent study showed that the function of SRC-1 is essential for the transactivation activity of progensterone receptor *in vitro* (42).

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Fig. 1

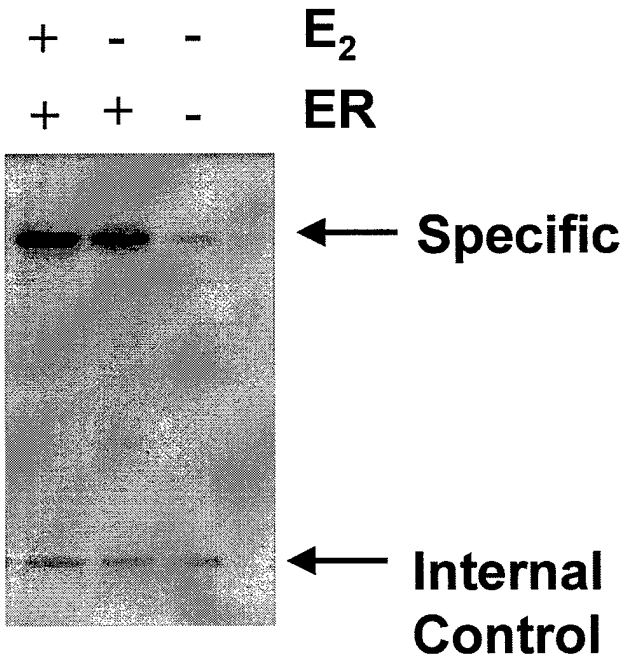


Fig. 2

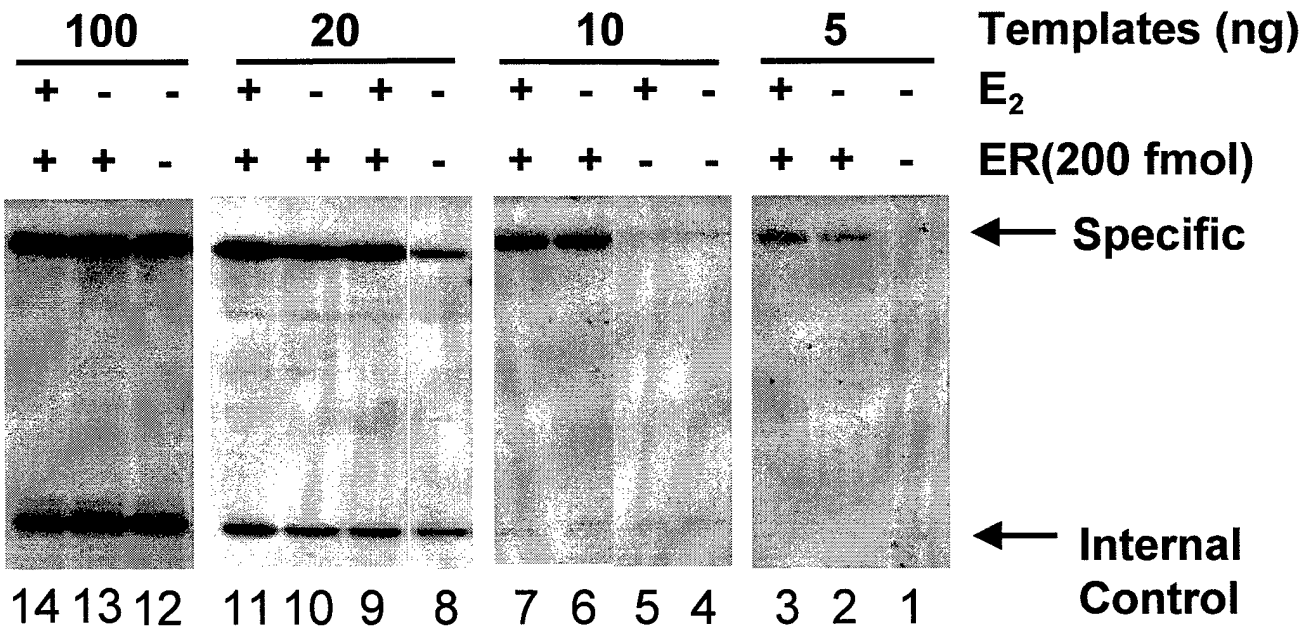
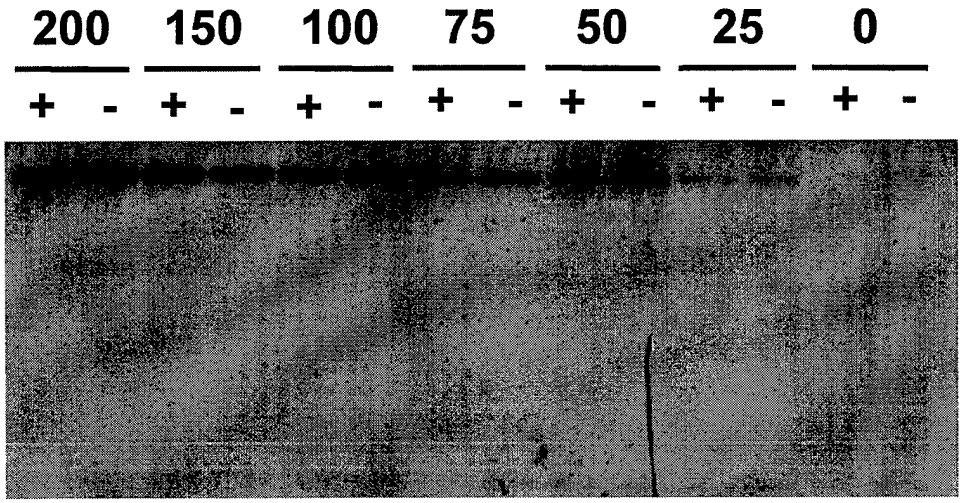

100
20
10
5
Templates (ng)
+ - - + - + - + - + - + - + - -
E_2
+ + - + + + - + + - - + + -
ER(200 fmol)
Specific
Internal Control
14 13 12 11 10 9 8 7 6 5 4 3 2 1

Fig. 3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ER
+ - + - + - + - + - + - + -
E_2
Specific
Internal Control
14 13 12 11 10 9 8 7 6 5 4 3 2 1

Fig. 4

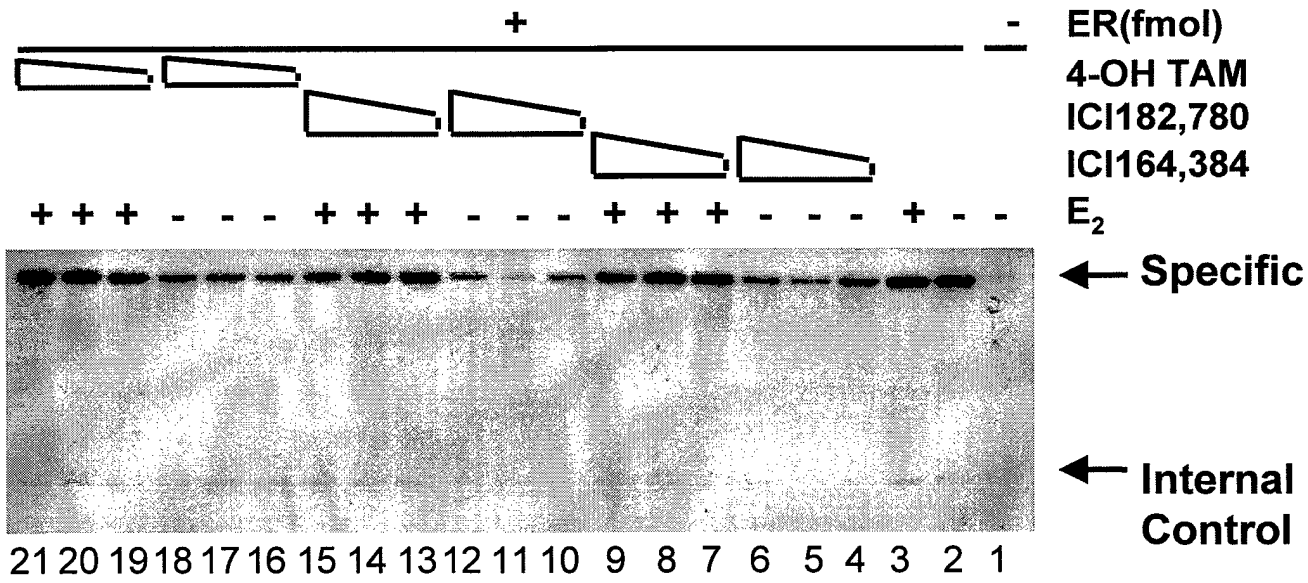

+
-
ER(fmol)
4-OH TAM
ICI182,780
ICI164,384
+ + + - - - + + + - - - + + + - - - + - -
E2
Specific
Internal Control
21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

Fig. 5

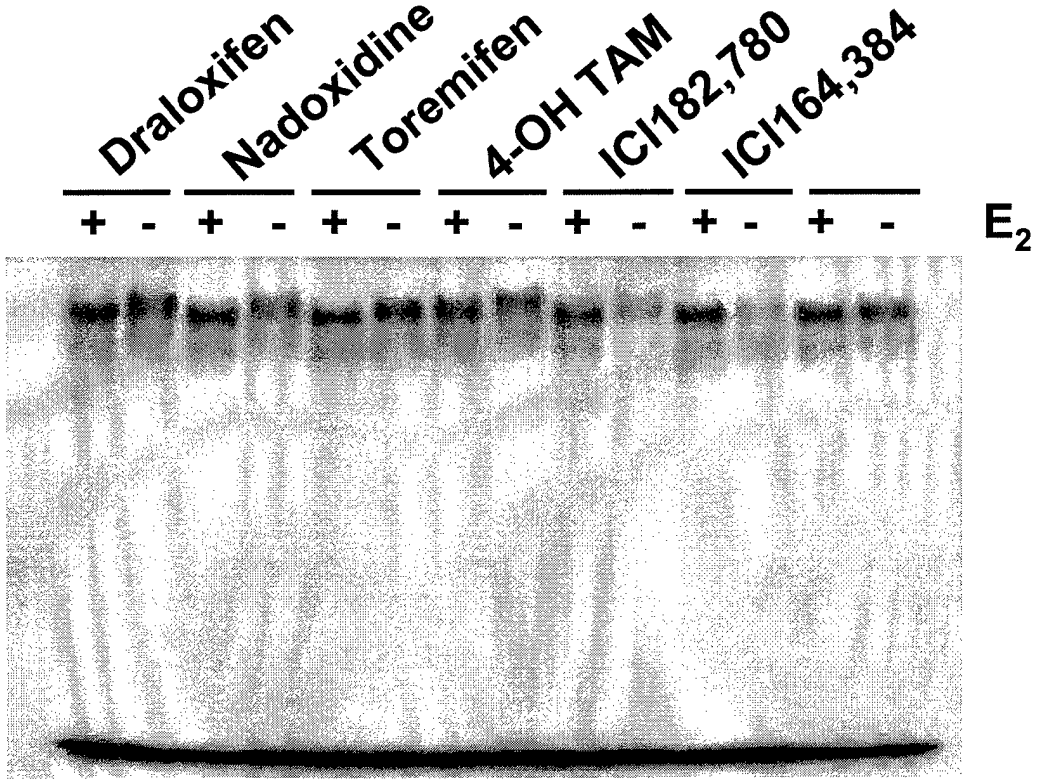

Draloxifen
Nadoxidine
Toremifen
4-OH TAM
ICI182,780
ICI164,384
+ - + - + - + - + - + - + -
E_2

Fig.6

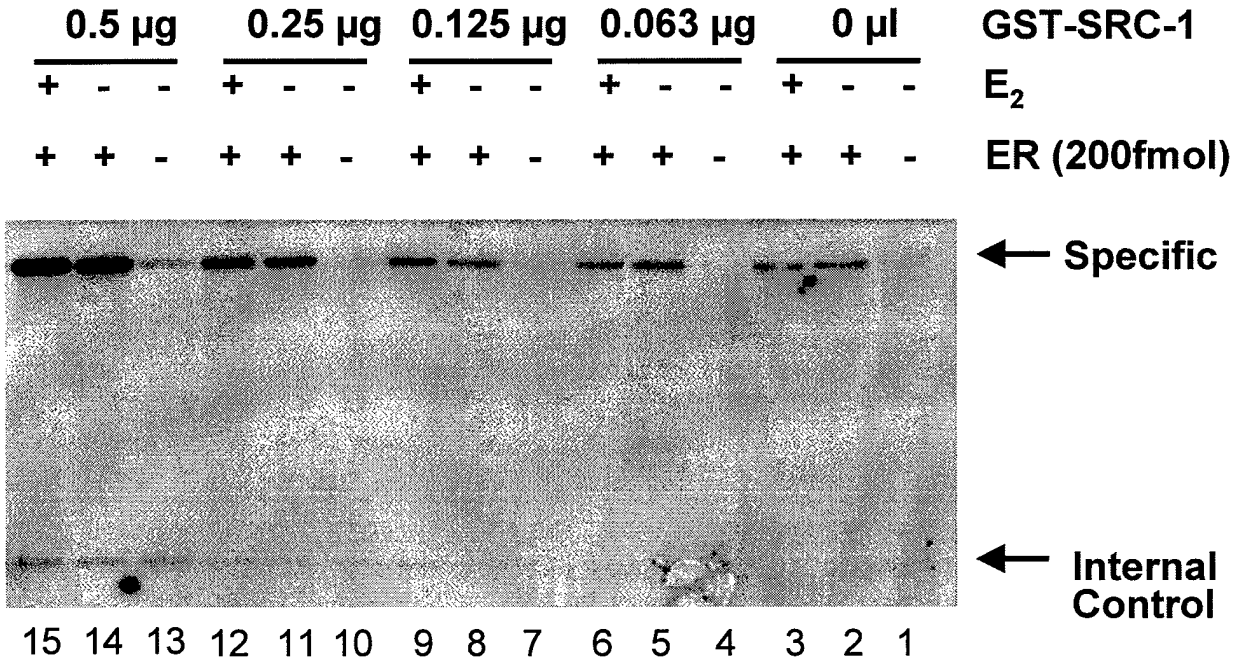

0.5 µg
0.25 µg
0.125 µg
0.063 µg
0 µl
GST-SRC-1
+ - - + - - + - - + - - + - -
E2
+ + - + + - + + - + + - + + -
ER (200fmol)
Specific
Internal Control
15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

Fig. 7

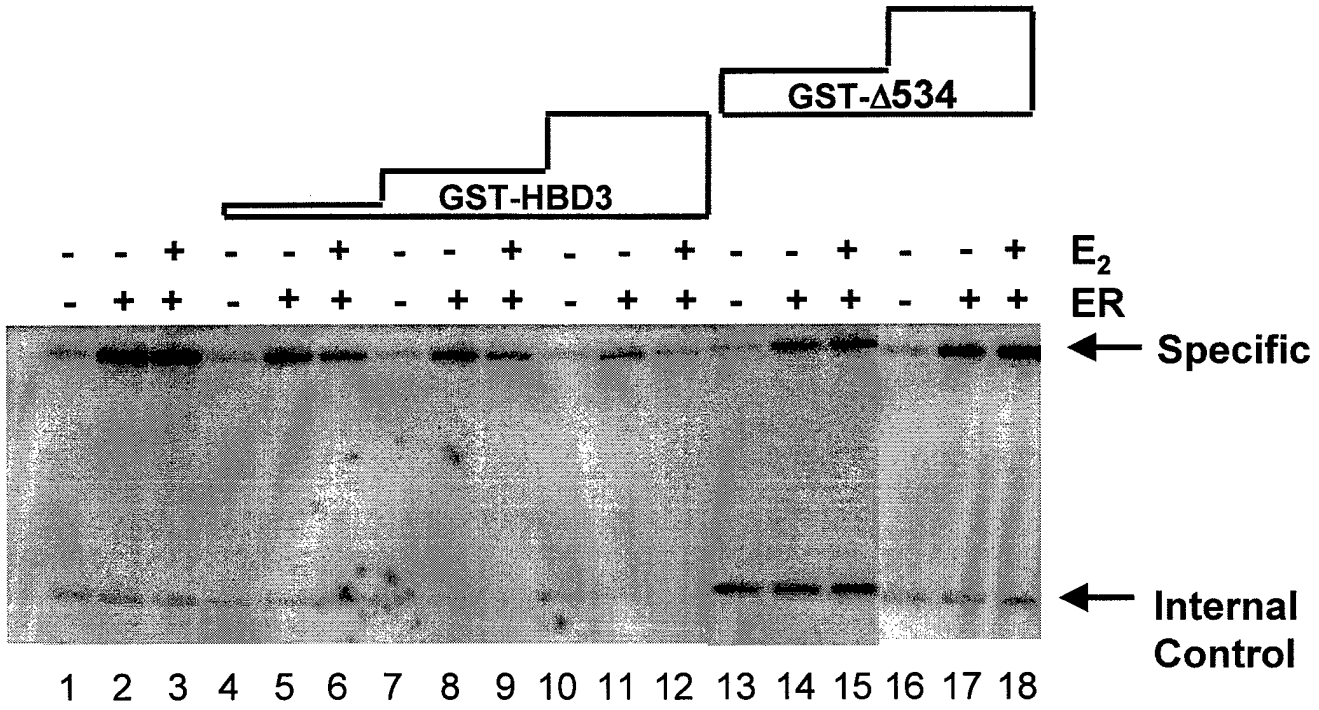

GST-Δ534
GST-HBD3
- - + - - + - - + - - + - - + - - +
E_2
- + + - + + - + + - + + - + + - + +
ER
Specific
Internal Control
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 8

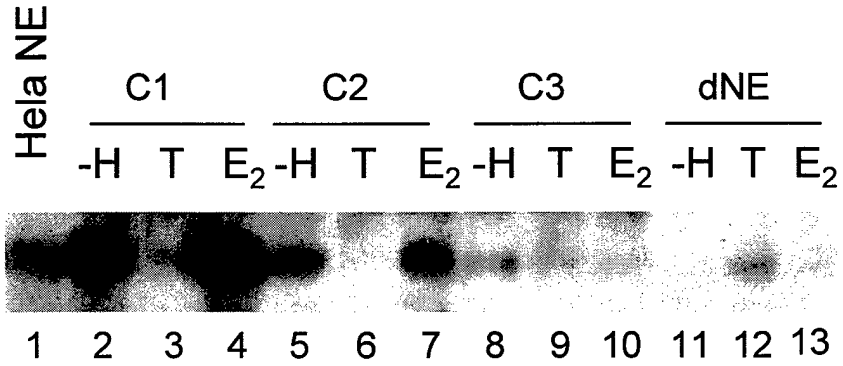
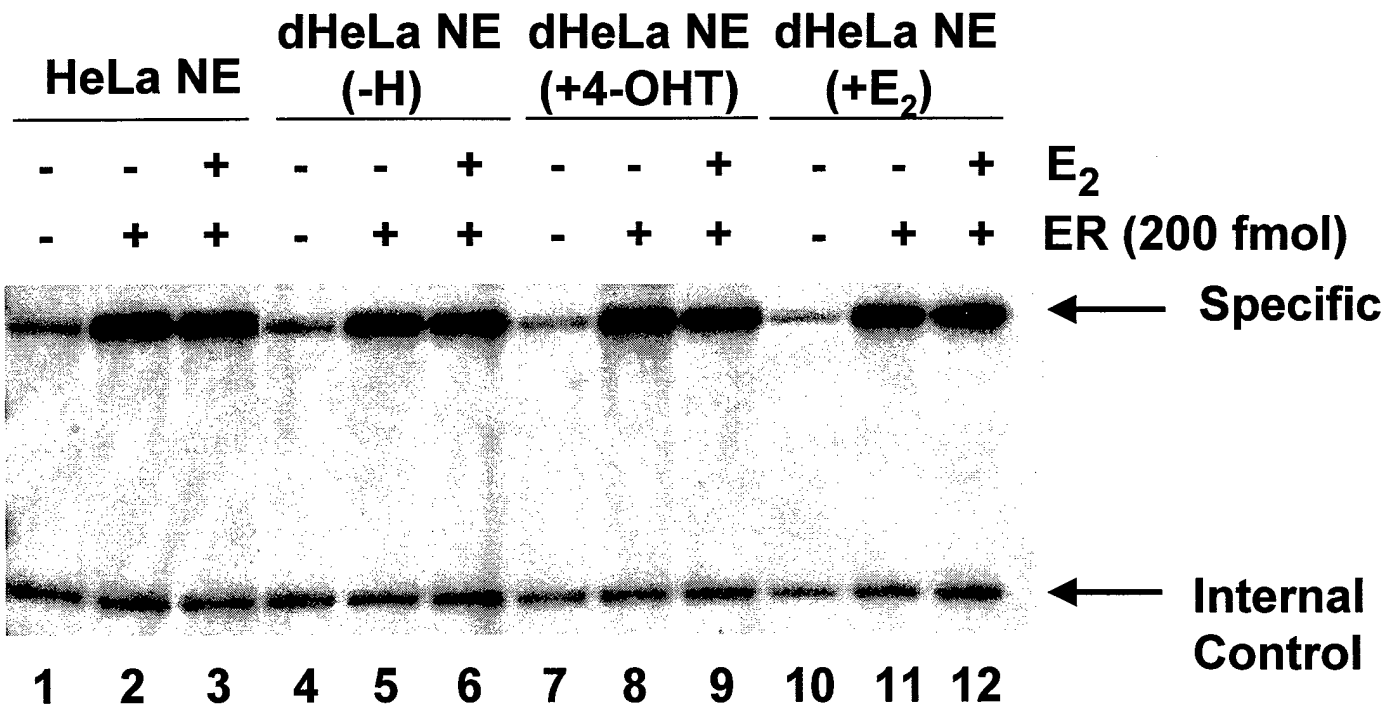


Fig. 9


HeLa NE
dHeLa NE (-H)
dHeLa NE (+4-OHT)
dHeLa NE (+E2)
- - + - - + - - + - - +
E2
- + + - + + - + + - + +
ER (200 fmol)
Specific
Internal Control
1 2 3 4 5 6 7 8 9 10 11 12